

Porcine insulin receptor substrate 4 (*IRS4*) gene: cloning, polymorphism and association study

Martin Masopust · Zuzana Vykoukalová ·
Aleš Knoll · Heinz Bartenschlager · Alan Mileham ·
Nader Deeb · Gary A. Rohrer · Stanislav Čepica

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Abstract Using PCR and inverse PCR techniques we obtained a 4,498 bp nucleotide sequence FN424076 encompassing the complete coding sequence of the porcine insulin receptor substrate 4 (*IRS4*) gene and its proximal promoter. The 1,269 amino acid porcine protein deduced from the nucleotide sequence shares 92% identity with the human IRS4 and possesses the same domains and the same number of tyrosine phosphorylation motifs as the human protein. We detected substitution FN424076:g.96C<G in the promoter region that segregates in Meishan and a synonymous substitution FN424076:g.1829T<C in the coding sequence with allele C present only in Meishan. Linkage mapping placed the *IRS4* gene at position 82 cM on the current USDA-USMARC linkage map of porcine chromosome X. Association analyses were performed on 555 animals of 12th–15th generation of the Meishan × Large

White cross and showed that both SNPs were highly significantly associated with backfat depth ($P = 0.0005$) and that the SNP FN424076:g.1829T<C was also associated with loin depth ($P = 0.017$). The Meishan alleles increased back fat depth and decreased loin depth. *IRS4* can be considered a positional candidate gene for at least some of the QTL located at the centromeric region of porcine chromosome X.

Keywords Pig · *IRS4* · PCR cloning · Polymorphism · Linkage mapping · Association analysis · SSCX QTL region

Introduction

Insulin receptor substrates (IRSs) play key roles in signal transduction from the insulin receptor as well as other receptors, including those for insulin-like growth factor I, growth hormone and some interleukins. Six members (*IRS1*, *IRS2*, *IRS3*, *IRS4*, *DOK4* and *DOK5*) of the IRS family have been identified that differ in some aspects including tissue distribution and developmental expression [1, 2]. The human *IRS4* gene encodes a 1,257 amino acid protein that contains, in order from its N-terminus, a pleckstrin homology (PH) domain, a phosphotyrosine binding (PTB) domain and 12 potential tyrosine phosphorylation (Tyr(P)) sites spread over the C-terminal region [3]. Fantin et al. [4] cloned the mouse *Irs4* gene, which encodes a 1,216 amino acid protein. Both the human and the mouse genes contain no introns in coding regions.

IRS4 is a non-abundant protein since it cannot be detected in any tissue by standard immunological methods [4]. A highly sensitive reverse transcription PCR method revealed that expression of *IRS4* mRNA is very low in

M. Masopust · A. Knoll · S. Čepica (✉)
Institute of Animal Physiology and Genetics AS CR, v.v.i.,
Liběchov, Czech Republic
e-mail: cepica@iapg.cas.cz

Z. Vykoukalová · A. Knoll
Mendel University in Brno, Brno, Czech Republic

H. Bartenschlager
Department of Animal Breeding and Biotechnology, University
of Hohenheim, Stuttgart, Germany

A. Mileham
Genus plc, 1425, River Road, DeForest, WI 53532, USA

N. Deeb
Genus/PIC, 100, Bluegrass Commons Blvd, Suite 2200,
Hendersonville, TN 37075, USA

G. A. Rohrer
USDA Agricultural Research Service, US Meat Animal
Research Center, Clay Center, NE, USA

different tissues. Although the IRS4 protein is expressed in heart and skeletal muscle it does not function as a substrate for the insulin and IGF1 receptors in primary human muscle cells [1, 5]. IRS4 is capable of mediating the phosphatidylinositol 3-kinase-dependent metabolic action of insulin in adipose cells and to promote the translocation of SLC2A4 that function as an insulin-regulated facilitative glucose transporter in cells of muscle and adipose tissue [6]. *Irs4* mRNA is highly expressed in the rat [7] and murine [8] hypothalamus suggesting a specific role for this factor in the signalling of one or more receptors involved in hypothalamic functions [7].

In Meishan × Western breed pedigrees, quantitative trait loci (QTL) for fat deposition, growth, carcass composition, food consumption, testis size and follicle stimulating hormone (FSH) level have been mapped to the centromeric region of porcine chromosome X (SSCX) and the data were deposited into PigQTLdb (<http://www.animalgenome.org/cgi-bin/QTLdb/SS/index> [9]). QTL for fat deposition, growth and muscling traits have also been detected in a European wild boar × Meishan (W × M) F₂ family [10] on the SSCX segment flanked by microsatellites SW2456 and SW1943. New QTL mapping on the W × M linkage map with seven gene tagged markers added between microsatellites SW259 and SW1943 showed that QTL for fatness, growth and most muscling traits mapped near *ACSL4* [11] located at position 80 cM on the current USDA-USMARC linkage map of SSCX [12]. The *IRS4* gene is located at 88.8 Mb on the SSCX physical map, about 0.6 Mb from the *ACSL* gene (88.2 Mb) (http://www.ensembl.org/Sus_scrofa/Info/Index/, assembly Scrofa9, September 2009). Milan et al. [13] and Duthie

et al. [14] located different QTL for fat accretion close to this map position.

The objectives of this study were to determine the DNA sequence of the porcine *IRS4* gene, detect polymorphisms in this sequence and perform an association study between polymorphisms of this gene and performance traits in an synthetic Meishan × Large White resource population.

Materials and methods

PCR cloning, sequencing, SNP detection

DNA was isolated from whole blood samples using phenol-chloroform extraction according to standard protocols. A fragment of the porcine *IRS4* was amplified with primer pair A (Table 1) designed from porcine EST sequence TC224218 obtained by blasting the human mRNA *IRS4* sequence NM_003604 against the porcine Gene Indices Project (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi>). The PCR product of predicted length was cut out of an agarose gel, cloned into *E. coli* and sequenced. PCR primers were designed using Oligo 4.0 Software [15]. The 5' and 3' ends of this sequence were extended by means of inverse PCR (IPCR) [16]. *Kpn*I cut porcine genomic DNA and primer pairs B and C were used to amplify the 5' flanking sequence, while the 3' flanking sequence was amplified using *Nhe*I cut porcine genomic DNA and primer pairs D and E (Table 1). The extended sequence was deposited in EMBL/GenBank/DDBJ databases. Polymorphisms were identified using comparative sequencing of PCR products prepared on template DNA

Table 1 Description of porcine *IRS4* primers

Primer	Sequence (5'-3')	Accession no.	Location of primers	Fragment size (bp)	MgCl ₂ (mM)	T _a (°C)
A	F: TACGAGAACGAGAAGAAAGTGG R: CGGCCGGAGGAAGCAGAGG	TC224218	110–130 3088–3105	3,397	1.5	63 ^a
B	F: GGCCTACTTCGTGCTCAAAC R: TAGCCCGTTGCAGACTTC	FN424076	778–798 732–751	–	2.0	56 ^a
C	F: AGCTCGGCTGGAATACTACCG R: ATGGCTCCCGGACAAGAC	FN424076	837–818 644–661	–	1,5	59 ^a
D	F: TTCCGCCCTCGCTCTTCTTCC R: CCGCCGTGCCGACCTTAG	FN424076	3752–3771 3886–3903	–	2.0	55 ^a
E	F: GCCGCCTCCTCTGGTC R: CTGGGGGAGAGAGAAATGTC	FN424076	3924–3941 3693–3712	–	1.5	60 ^a
I	F: TCGCCCCCTCCAGCCAAT R: TCGGAGAAGGGAGGGTTGGG	FN424076	4–21 437–456	453	1.5	64 ^a
J	F: CAGGCAGGAAGACTGCACCT R: GCCATTCTGAGCCCCACTT	FN424076	1727–1747 1974–1994	271	1.0	55

T_a annealing temperature, F forward primer, R reverse primer

^a LA polymerases mix (Top-Bio, Prague, Czech Republic) was used instead of *Taq* polymerase

from Meishan, wild boar, Piétrain, Piétrain × wild boar and Piétrain × Meishan pigs. Domains of the deduced protein were detected using InterProScan software [17].

Genotyping and linkage mapping

PCR-RFLP genotyping assays for SNP FN424076:g.96C>G and SNP FN424076:g.1829T>C used *Fnu*4HI and *Taq*I restriction enzymes and primer pairs I and J, respectively. For linkage mapping the USDA-USMARC backcross family [18] and larger wild boars × Meishan (W × M) and Meishan × Piétrain (M × P) populations [19] were used. Multipoint linkage analysis of the families was performed using CRI-MAP version 2.4 [20]. Linkage disequilibrium (LD) measures were calculated using 2LD software [21].

Association analysis

Animals

Association analyses of SNP FN424076:g.96C>G and FN424076:g.1829T>C were performed on 555 animals (317 male and 238 female animals) and 554 animals (315 male and 239 female animals), respectively, of 12th–15th generation of the Meishan × Large White (MLW) cross (PIC, Hendersonville, USA) with records for weight at the end of the test, lifetime daily gain, test time daily gain, loin depth (measured at the 10th rib with the skin removed) and backfat depth (measured from the top to the bottom of the loin at the 10th rib). Means and standard deviation of traits are given in Table 2. The MLW cross is more suitable for fine association mapping of QTL than an *F*₂ population because of it's a shorter extent of LD [22, 23].

Statistical analyses

Associations between *IRS4* genotypes and traits were analysed using the GLM procedure of SAS, release 8.2

Table 2 Overall means and phenotypic standard deviation (SD) of the five traits studied in Meishan × Large White (MLW) cross

Trait	N ^a	Mean	SD	Minimum	Maximum
Age at slaughter (days)	565	144.91	3.98	134.00	155.00
Weight at the end of test (kg)	565	81.31	10.34	60.80	119.30
Lifetime daily gain (g)	565	560.69	65.90	417.93	784.87
Test time daily gain (g)	565	781.02	110.44	457.90	1155.88
Loin depth (mm)	565	45.07	6.24	26.12	68.68
Backfat depth (mm)	565	16.88	4.17	5.94	36.98

^a Genotyping of some animals was not successful and therefore numbers of animals in association tests are different

(SAS Institute Inc., Cary, NC, USA). The effects of *IRS4* genotype were calculated according to the following equation:

$$Y_{ijkl} = \mu + \text{sex}_i + \text{IRS4}_j + \text{year_s}_k \\ + b(s_{\text{age}}_{ijkl} - S_{\text{age}}) + e_{ijkl}$$

where Y_{ijkl} = value of the dependent trait; μ = estimated mean value of the dependent trait; sex_i = fixed effect of *i*th sex, male, female; IRS4_j = fixed effect of the *j*th genotype of *IRS4* locus, three genotype classes of locus *IRS4* (either *C/C* and *C/–*, *C/G*, *G/G* and *G/–* for FN424076:g.96C>G or *T/T* and *T/–*, *T/C*, *C/C* and *C/–* for FN424076:g.1829T>C); year_s_k = fixed effect of *k*th year season of the test (3 months each); b = linear regression value; s_{age}_{ijkl} = slaughter age of an animal; S_{age} = estimated average of the slaughter age; e_{ijkl} = residual value. P values were corrected for multiple testing [24]. PV% is the percentage of the error variance reduction by including genotype information in the initial environmental model without the marker effect. The additive (*a*) and dominance (*d*) effects were defined as the deviation from the mean of the two homozygotes of animals homozygous for the *96G* or *182C* allele or animals heterozygous in the loci *96C>G* or *1829T>C*, respectively. If there is no dominance, *d* = 0, if the allele that increases the value is dominant over the other allele, *d* is positive, and if the allele that decreases the value is dominant over the other, *d* is negative [25].

Results and discussion

PCR cloning and sequencing

Using PCR and IPCR techniques we obtained the 4498 bp nucleotide sequence FN424076 encompassing the complete coding sequence of the porcine *IRS4* gene and its proximal promoter (Fig. 1). The complete coding sequence of porcine *IRS4* gene is located in a single exon without any introns. The porcine *IRS4* coding sequence shares 87 and 79% identity with the human (NM_003604) and mouse (NM_010572) *IRS4* coding sequences, respectively. The porcine 1,269 amino acid protein (CAZ66650.1) deduced from the nucleotide sequence FN424076 shares 92% identity with the 1,257 amino acid of the human IRS4 (AAC51738.1) and possesses the same domains and the same number of (Tyr(P)) motifs as the human protein [3]. The PH domain of porcine IRS4 consists of 120 amino acids (residues 78–197) and exhibits a high degree of homology with PH domain of the human IRS4 (99% identity). The PTB domain of porcine IRS4 consists of 101 amino acids (residues 231–331) and exhibits 100% identity with the PTB domain of the human IRS4. Comparison of

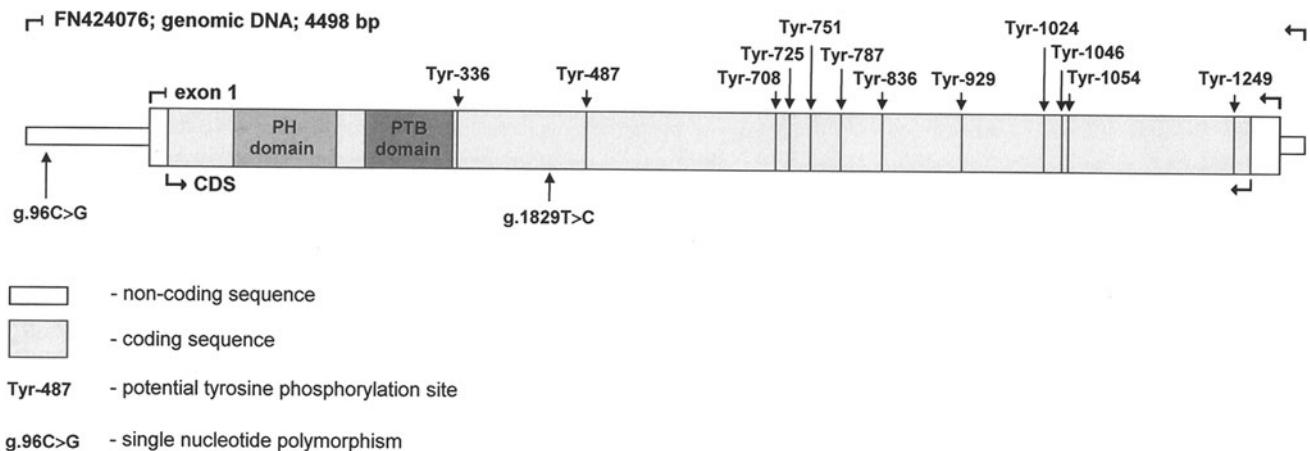


Fig. 1 Scheme of the porcine *IRS4* gene

the porcine protein sequence (CAZ66650.1) with the human [3] and mouse [4] proteins revealed 12 potential (Tyr(P)) sites spread over the C-terminal portion of the porcine IRS4 protein (Fig. 1), seven of which are in YXXM motifs (Tyr 487, 708, 725, 751, 787, 836 and 1,249). This motif binds to the SH2 domain of the PIK3R (phosphoinositide-3-kinase, regulatory subunit 1 (alpha)), which plays an important role in the metabolic actions of insulin (GeneID:5295).

Polymorphisms

Comparative sequencing revealed a substitution in the promoter region (FN424076:g.96C>G) detectable by *Fnu4HI* and a synonymous substitution FN424076:g.1829T>C in the coding sequence, which can be genotyped using *FaqI*. SNP 96C>G was genotyped in 53 unrelated animals from six breeds: Czech Large White ($n = 10$), Czech Landrace ($n = 10$), Black Pied Prestice ($n = 10$) Duroc ($n = 10$), Hampshire ($n = 4$), Meishan ($n = 8$) and wild boar ($n = 1$). The G allele was detected only in Meishan with frequency 0.56 while in other breeds and wild boar only the C allele was observed. SNP 1829T>C was genotyped in 137 unrelated animals from 8 breeds, Czech Large White ($n = 28$), Czech Landrace ($n = 27$), Piétrain ($n = 19$), Black Pied Prestice ($n = 21$), Czech Meat Pig ($n = 14$), Duroc ($n = 10$), Hampshire ($n = 6$), and Meishan ($n = 13$). The C allele was fixed in Meishan while the T allele was fixed in the rest of breeds with exception of Landrace where the frequency of the C allele was 0.04 ($n = 27$). In the MLW the loci are in nearly complete LD ($D = 0.1396$ and $D' = 0.9868$).

Linkage mapping

Linkage mapping placed the *IRS4* gene on basis of 77 informative meioses at position 82 cM on SSCX on the current USDA-USMARC linkage map [7] with gene order

ACSL4 (80.0 cM)—*CAPN6* (81.0 cM)—*IRS4* (82 cM)—*PAK3* (82.5 cM). Mapping on the W × M (668 informative meioses) and the M × P (630 informative meioses) families put the *IRS4* between *ACSL4* and *CAPN6* gene at positions 91.7 and 74.2 cM, respectively, on the corresponding linkage maps, which matches position 80.2 cM on the current USDA-USMARC linkage map of SSCX. In humans the gene order is *IRS4* (107.97 Mb)—*ACSL4* (108.88 Mb)—*PAK3* (110.18 Mb)—*CAPN6* (110.48 Mb) (http://www.ensembl.org/Homo_sapiens/Info/Index—assembly GRCh37, January 2010) while the gene order on the contemporary pig physical map is *PAK3* (77.8 Mb)—*IRS4* (88.2 Mb)—*ACSL4* (88.8 Mb)—*CAPN6* (89.1 Mb) (http://www.ensembl.org/Sus_scrofa/Info/Index—assembly Sscrofa9, September 2009). There is very high conservation of gene order between the human physical map and the pig clone map of chromosome X, except for two small segments. The chromosome segment encompassing the *IRS4* gene is one of two exceptions with a possible mistake in the pig sequence assembly [26]. The peaks of QTL for fat accretion traits, muscling and growth traits were mapped with high statistical power and precision [27] on the Meishan × Large White reference population with more than 1,100 F₂ pigs to an average position of 80.5 cM, ranging from 77 to 84 cM for different traits [13] and to position 80 cM on the high density gene based linkage map of Hohenheim wild boar × Meishan F₂ reference population [11]. QTL for testis size and FSH concentration were mapped approximately to the same position as the QTL for fat accretion [28, 29].

Association analysis

Frequencies of the 96G and 1829C alleles in the MLW were 0.20 and 0.31, respectively. The 96C>G and 1829T>C loci were highly significantly associated with backfat depth (Table 3) with additive effects $a = 1.39 \pm 0.25$ mm ($P < 0.0001$) and $a = 1.40 \pm 0.22$ mm ($P < 0.0001$) and

Table 3 Associations of the FN424076:g.96C>G and FN424076:g.1829T>C within the *IRS4* gene with phenotypic traits in Meishan × Large White (MLW) cross

SNP/Trait	F test		PV (%)	LS means ± SE	P (t test)			
	F value	P value			C/C + C/I−	C/G	G/G + G/I−	C/C + C/I− vs. C/G
FN424076:g.96C>G								
Weight at the end of test (kg)	1.56	0.2142		N = 410	N = 65	N = 80		
Lifetime daily gain (g)	1.55	0.2142		82.49 ± 0.60	84.73 ± 1.24	82.00 ± 1.13	0.0906	0.1111
Test time daily gain (g)	1.81	0.2142		568.61 ± 4.12	583.89 ± 8.57	564.95 ± 7.82	0.0942	0.1099
Loin depth (mm)	2.80	0.1543		784.92 ± 7.22	815.39 ± 15.03	787.09 ± 13.54	0.0580	0.1708
Backfat depth (mm)	14.83	0.0005***	4.86	46.81 ± 0.38	45.38 ± 0.79	45.56 ± 0.72	0.0901	0.8696
				16.47 ± 0.27	16.78 ± 0.55	19.24 ± 0.50	0.5936	0.0014***
FN424076:g.1829T>C								
Weight at the end of test (kg)	0.16	0.8611		N = 329	N = 102	N = 123		
Lifetime daily gain (g)	0.14	0.8611		82.70 ± 0.66	83.21 ± 1.07	83.11 ± 0.96	0.6755	0.9502
Test time daily gain (g)	0.53	0.8611		569.97 ± 4.56	573.40 ± 7.40	572.53 ± 6.66	0.6806	0.9337
Loin depth (mm)	4.23	0.0170*	1.18	787.07 ± 7.98	793.23 ± 12.98	798.69 ± 11.50	0.6736	0.7647
Backfat depth (mm)	21.20	0.0005***	6.95	47.17 ± 0.42	45.61 ± 0.68	45.76 ± 0.61	0.0421*	0.8753
				16.08 ± 0.29	16.83 ± 0.47	18.88 ± 0.42	0.1490	0.0020**
								<0.0001***

For each genotype, the estimated least square means using GLM procedure and the SE are given

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, P is P value adjusted for multiple testing [24], PV (%) is the percentage of the error variance reduction by including genotype information in the initial model without genetic component

dominance effects $d = -1.07 \pm 0.63$ mm (non-significant (NS)) and $d = -0.65 \pm 0.55$ (NS). The *I829T>C* locus was also associated with loin depth with an additive effect $a = -0.70 \pm 0.32$ mm ($P = 0.0274$) and dominance effect $d = -0.85 \pm 0.81$ (NS). Differences between contrasting genotypes were equal to 0.66 and 0.67 SD for backfat depth at the *96C>G* and *I829T>C* loci, respectively, and 0.23 SD for loin depth at the *I829T>C* locus.

Female mice lacking *Irs4* were less fertile than wild type. *Irs4* knockout in mice caused mild defects in growth, reproduction, and glucose homeostasis [30]. Mice with neuron specific deletion of the insulin receptor display increased body fat content and hypothalamic impairment for reproduction [31]. Recently it has been shown that IRS4 couples to the leptin receptor. Both leptin and insulin regulate energy expenditure and glucose homeostasis by acting on hypothalamic neurons, and cross talk between the insulin and leptin signal transduction pathways is well documented. Both hormones can activate the insulin receptor substrate—phosphatidylinositol 3-kinase pathway in the hypothalamic arcuate nucleus [32] that participates in the control of food intake, long-term control of adiposity [33], secretion of gonadotropin [34] and regulation of reproduction [35, 36].

The functions of the *IRS4* gene indicate that polymorphisms in the gene could explain associated effects. Of the two SNPs that are in nearly complete LD, the synonymous SNP *1829T>C* is an obvious candidate because of higher *F* value and higher percentage of PV (Table 3). The mouse *Irs4* gene is predicted to be targeted by 31 miRNAs (<http://mirtab.org/miRDB>), which are an important class of gene regulators. Thus, a SNP within the porcine gene could disrupt a miRNA target or a SNP could be located within the sequence coding for miRNA [37]. *In silico* searches for miRNA targets will be possible after sequencing of the porcine genome is finished.

If the studied SNP were not causative then an association between a marker and phenotypic traits would be based on LD. One of the main sources of LD in farm animal populations is crossbreeding while LD is mainly eroded by recombination [22]. For tightly linked loci, any LD that has been created will persist over many generations but, for loosely linked loci, LD will decline rapidly over generations. In this study, phenotypes were measured in 12th–15th generation of the MLW cross. The extent of LD in the Meishan line brought to Europe about 25 years ago (small number of founders) is higher compared to that in other Chinese breeds, although still lower than that in most European breeds. In Chinese pig breeds, LD is mostly organized in haploblocks of up to 10 kb, while in European breeds, LD haploblocks may be up to 400 kb in size [23]. Useful LD ($r^2 > 0.3$) for detectable association is between 0.1 and 2 cM in European breeds and between 0.005 and

0.05 cM for Chinese breeds, provided that 1 cM is equivalent to 1 Mb [23]. The detection of highly significant associations of *IRS4* SNPs with effects on backfat depth of the same direction and magnitude as detected in *F₂* populations (<http://www.animalgenome.org/cgi-bin/QTLdb/SS/index>, [9]) suggests that the QTL and *IRS4* are less than 2 cM apart from each other.

Based on map position and function, in addition to *AR* [38], *SERPINA7* [39, 40], and *ACSL4* [11, 41] genes, *IRS4* can be considered a positional candidate gene for at least some of the QTL located at the centromeric region of SSCX.

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